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Novel Peptidomimetics:[1] Inhibitors of Substance P Endopeptidase

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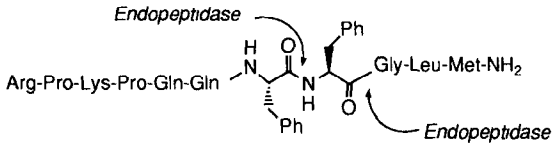
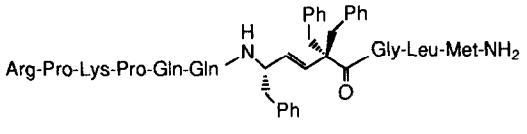
Abstract: The synthesis of the novel *bis*-phenylalanine mimetic **1** and its incorporation into substance P are described. The key step in the synthesis of **1** is a Julia olefination reaction resulting in a *trans*-olefin. The synthesized compounds were able to inhibit a human substance P endopeptidase but lacked appreciable affinity for the rat NK_1-receptor.

Substance P (SP), a linear undecapeptide of the tachykinin family,[2] is involved in several important physiological processes such as neurogenic inflammation and pain transmission.[3] The physiological effects of SP are mediated by its activation of NK_1-receptors.[4] SP is unstable *in vivo* due to efficient degradation by specific peptidases. One such enzyme is substance P endopeptidase (SPE), a converting enzyme isolated from human cerebrospinal fluid, which predominantly catalyzes the hydrolysis of the Phe-Phe amide bond and to a minor extent the Phe-Gly bond.[5] Access to inhibitors of SPE would make it possible to modulate SP tonus and would also enable detection of SP *in vivo*.

We now report on the synthesis of the *bis*-phenylalanine mimetic **1** and its incorporation into SP giving compound **2**. Compound **1** was designed as a metabolically stable replacement of the major enzymatic cleavage site in SP.[6] Three advantages of derivative **1** over a true vinyl Phe-Phe isostere were identified; (i) the peptidomimetic **1** only possesses one stereogenic centre, thus simplifying the synthesis, (ii) the double bond cannot isomerize into conjugation with the carbonyl group and thereby changing the geometry around the replaced amide bond position, and (iii) the additional benzyl group might prevent cleavage also between Phe-Gly in **2** due to the steric bulk of one of the benzyl groups which shields the amide bond.



Substance P



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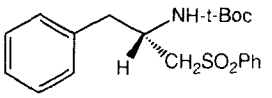
Synthesis The key step in the synthesis of **1** is a Julia olefination reaction using the sulfone **3** and the aldehyde **4**.[7] The enantiopure sulfone was prepared from *t*-butyloxycarbonyl (Boc) protected L-phenylalanine by use of a modification of a literature procedure.[8,9] The optical purity (> 99%) was determined indirectly (^{19}F-NMR) after deprotection of the Boc group followed by amidation using *(S)*-Mosher acid chloride.[10] Aldehyde **4** was synthesized from dimethyl malonate. Dibenzylation was achieved by treatment of dimethyl malonate with K_2CO_3 and an excess of benzyl bromide. $LiAlH_4$-reduction produced the *bis*-alcohol which was *mono*-silylated with *t*-butyldimethylsilyl chloride. Swern oxidation[11] (oxalyl chloride, DMSO) proceeded in good yield and completed the synthesis of **4**.



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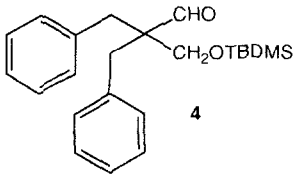

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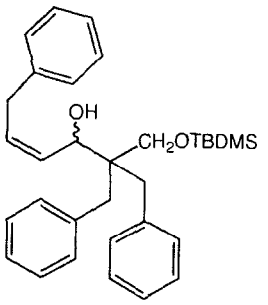
The Julia olefination reaction which produced the *trans*-olefin **7** (Scheme 1) involves three consecutive steps, (i) the aldehyde is activated by complexation with DIBAL methoxide, (ii) the coupling is performed by addition of the aldehyde-DIBAL complex to the lithium salt of the sulfone and (iii) a concomitant elimination/desulfonylation[12] step achieved by treatment of the coupling product with amalgamated sodium (6%). The overall yield of **7** was 40 %. In the elimination/desulfonation reaction also 6 % of a byproduct, tentatively assigned as **5** was isolated. It is tempting to suggest that **5** and **7** might be formed from different diastereomers of intermediate **6**; the direction of elimination may be dependent on the relative stereochemistry of the stereogenic centra formed in the condensation of **3** and **4**.



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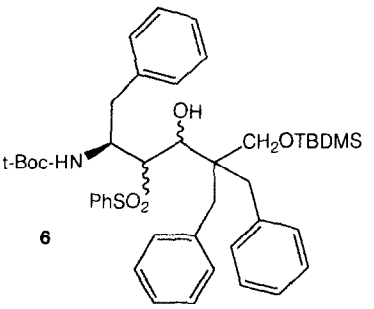
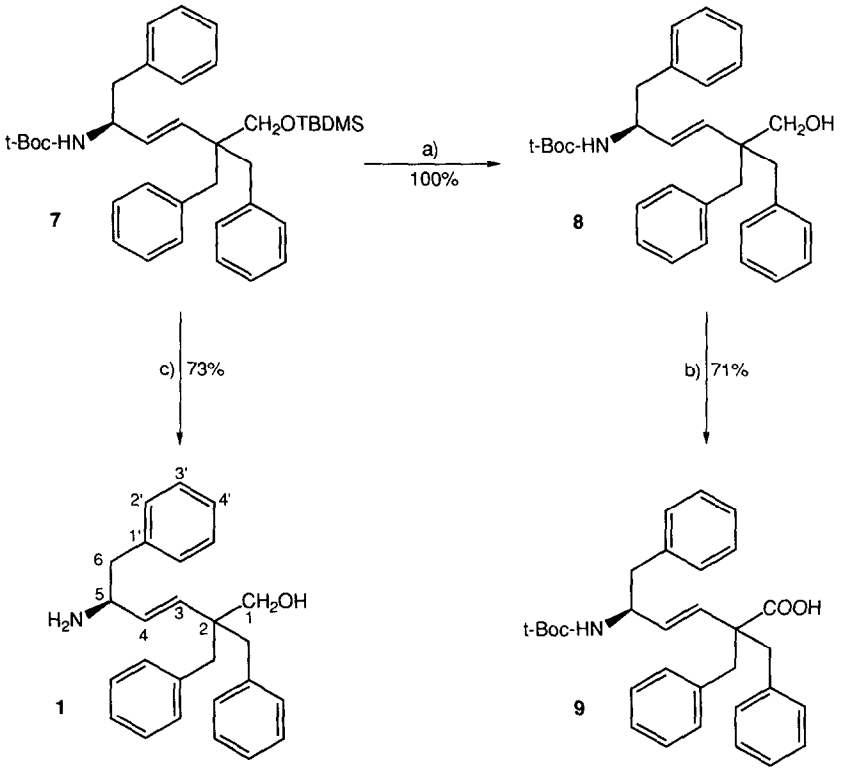


6

Deprotection of **7** to form **1** in 71 % yield was performed in one step by treatment with HF in CH_3CN. Before characterization and testing the compound was converted to its hydrochloride salt.[13] To enable incorporation of fragment **1** into SP the *t*-Boc protected acid derivative was required. Desilylation of **7** by tetrabutylammonium fluoride[14] gave the alcohol **8** which was oxidized by Jones' reagent to afford **9** in 73 % yield.

Scheme 1



Reagents: a) Bu4NF, THF, 22°C, 2h; b) Jones' reagent, 0°C, 1h; c) 40% HF in CH_3CN, 22°C, 0.5h.

Compound **2** was synthesized by the solid-phase method[15] on an Applied Biosystems 430 A instrument using Boc/Bzl protecting groups and procedures essentially as recommended by the manufacturer. However, double couplings were used and capping with acetic anhydride was included at the end of each reaction cycle. Compound **9** (1.5 eq) and the Boc-glutamine in position 6 (3 eq) were coupled manually using 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU) in the presence of diisopropylethylamine and 1-hydroxybenzotriazole.[16]

The product obtained after HF-cleavage was purified by reversed phase HPLC on a Pharmacia Pep-RPC column (10 x 1 cm) with a 0.1 % trifluoroacetic acid (TFA)/CH_3CN gradient (20 to 40 % CH_3CN in 40 min) at a flow rate of 2 mL/min. Time-of-flight plasma desorption mass spectrometry[17] gave an m/z value of 1422.3±1.4 for the $[M+H]^+$ peak (calculated Mw = 1422.0). No peaks were observed at m/z values corresponding to the Met(O) derivative or to truncated peptides.[18] The ^{1}H-NMR spectrum shows a vicinal coupling constant of the vinylic protons of 16.1 Hz which is indicative of a *trans* olefin.

SPE Inhibition. The SPE was purified from human cerebrospinal fluid following the procedure described previously[5] and its activity was determined by monitoring the conversion of SP into its *N*-terminal fragment

SP(1-7) by reversed phase chromatography using a SMART system[19] (Pharmacia LKB Biotechnology, Uppsala, Sweden). Incubations were performed in Eppendorf tubes at 37°C with 2 nmol (40 μM) SP and 1 μg of the purified CSF enzyme together with the inhibitor in a final volume of 50 μL Tris-HCl buffer (40mM, pH 7.8). Prior to the addition of the substrate the enzyme was preincubated with the inhibitor for 20 min at 37°C. The incubation proceeded for 30 h and aliquots for analysis were frozen at -70°C to terminate the reaction. The aliquots were diluted (1 : 1) with 0.04% TFA and analyzed by the SMART system.[20] The identity of the separated SP fragments was confirmed by FAB mass spectrometry on a Finnegan MAT 90 instrument as described earlier.[21]

Figure 1 shows the time-course of the SPE-catalyzed release of SP(1-7) from SP in the absence and presence of inhibitors. The substrate concentration was 40 μM and the inhibitors were added in a 4.5-fold excess. The substrate concentration was kept high due to the particular detection system used. As shown in Figure 1 both agents strongly depressed the SPE activity. Additional studies indicated that 50% inhibition of the enzyme activity was obtained at inhibitor concentrations of 1.5 μM and 10 μM of **1** and **2**, respectively.

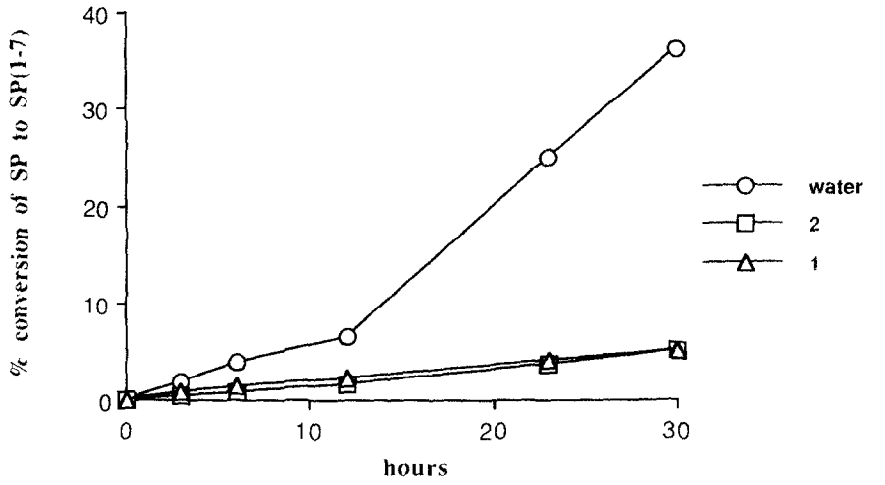


Figure 1. Time course for the generation of SP(1-7) from SP by SPE as recorded by reversed phase HPLC on a SMART system. The SP concentration was 40 μM and the inhibitor concentration 180 μM.

NK1-Receptor binding. Compounds **1** and **2** were also tested in an NK1 receptor binding assay. Membranes were prepared from whole rat brain minus cerebellum. The tissue was homogenized in 10 vol. 0.32 M sucrose in the cold. The homogenate was centrifuged at 1000 x g for 10 min and the supernatant was saved and centrifuged at 18 000 x g for 20 min at 4°C. The pellet was saved, thoroughly mixed with 4 vol distilled water and incubated for 8 min at 25°C to cause lysis. The lysate was centrifuged at 10 000 x g for 20 min at 4°C and the supernatant with membranes was saved. The membrane suspension was diluted with 50 mM Tris-HCl buffer, pH 7.4 and pelleted by centrifugation. The membranes were resuspended in a small volume of Tris-HCl.

Binding assays were run essentially as described by Tousignant *et al.*[22] About 10 000 cpm of [^{3}H]Ser^9, Met(O$_2$)^{11}-substance P and 0.75 mg of membrane protein were incubated at 25°C for 30 min in 200 µL of a medium containing 50 mM Tris-HCl (pH 7.4), 3 mM $MnCl_2$, 2 mg/mL bovine serum albumin, 40 µg/mL bacitracin, 4 µg/mL phosphoramidon. Incubations were terminated by filtration through Whatman GF-C membranes presoaked in 0.3% polyethyleneimine solution for at least 3 h at 4°C. Each assay included triplicates of all samples with competitor and of two controls. Compounds **1** and **2** were tested in at least three separate experiments, however none of the compounds showed any ability to displace the radioligand from the NK$_1$-receptor even at µM concentrations.

Conclusion: The present data show that substitution of the *bis*-phenylalanine moiety in substance P with a non-peptidergic fragment produces an inhibitor of SPE. Even the dipeptidomimetic itself produces enzyme inhibition. However, the change in the peptide structure does prevent NK$_1$-receptor recognition and binding. Ongoing studies focus on establishing the selectivity of **1** and **2** for SPE and on the synthesis of additional dipeptidomimetics.

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13. Data on **1**: ^{1}H-NMR ($CDCl_3$): δ 2.39 (s, 2H, benzylic), 2.46 (d, 1H, benzylic), 2.91-2.99 (m, 2H, H6´, benzylic), 3.20-3.35 (m, 3H, H1, H1´, H6), 3.89 (ddd, 1H, H5), 5.56 (m, 2H, H3, H4), 6.58-7.32 (m, 15H, aromatic); ^{13}C-NMR ($CDCl_3$): δ 39.37, 39.55, 41.59 (C6, CH_2Ph), 45.25 (C2), 56.71 (C5), 64.64 (C1), 124.90 (C3), 126.05, 127.10, 127.82 (C4´), 127.82, 127.91, 128.68, 129.74, 130.62, 131.01 (C2´, C3´), 135.86, 136.80, 137.24 (C1´), 143.65 (C4); mp: 96-99°C, $[\alpha]_D$ = -33.5° (*c* = 1.07, $CHCl_3$).
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18. Selected data on **2**: ^{1}H-NMR (CD_3OD): vinylic protons δ 5.58 (dd, 1H, *J* 16.1 and 5.4 Hz) 5.78 (d, 1H); ^{13}C-NMR (CD_3OD): vinylic carbons δ 132.30 and 133.84; $[\alpha]_D$: -43.2° (*c* 0.5, CH_3OH); HPLC retention time: On analytical RP-HPLC (Pep-RPC, 5 x 0.5 cm) using a linear gradient of CH_3CN in 0.1% TFA (0 to 60 % CH_3CN in 30 min) at a flow rate of 1 mL/min the elution volume of **2** was 19.2 mL.
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